

DESCRIPTION

5 METHOD FOR PREDICTING FORMATION OF SPOTS USING A GENE
GROUP EXHIBITING INCREASED EXPRESSION AT A SPOT SITE
AS AN INDICATOR AND SCREENING METHOD FOR
SPOT FORMATION INHIBITORS

TECHNICAL FIELD

10 The present invention relates to a skin test method
for predicting the formation of spots.

BACKGROUND ART

15 When the action of the enzyme tyrosinase within
melanocytes (pigment-forming cells) is activated
abnormally due to ultraviolet rays, hormonal imbalance or
psychological stress and so forth, formation of melanin
pigment is enhanced and they are successively sent out to
surrounding epidermal cells. If the rate at which
melanin pigment is produced is excessively fast and
turnover is no longer normal due to the effects of
20 ultraviolet rays and so forth, the melanin pigment is
unable to be excreted to the outside and remains in the
skin, and this is believed to result in the formation of
spots in the skin.

25 Once a spot has been formed, it is preferably to
treat the spot as quickly as possible, and a visual
sensory evaluation of the spot by a beautician, or an
early assessment of the presence of a spot by a
quantitative evaluation of the spot using equipment such
as an apparatus for capturing images of skin condition or
30 a colorimeter, is desired for the purpose of providing
treatment (Japanese Unexamined Patent Publication No.
2003-144393).

35 Once a spot being formed, it is not easily removed,
and treatment is required that improves skin metabolism
to quickly expel the unnecessary melanin and prevent
excess melanin from being formed. Thus, it is preferably
to care for the skin prior to the formation of a spot.

However, since there are individual differences in susceptibility to spot formation and there are various conditions that cause their formation, it is typically difficult to predict the formation of a skin spot.

5 Accordingly, a means for predicting whether or not the skin is susceptible to spot formation prior to formation thereof would be extremely effective as a preventive measure.

DISCLOSURE OF THE INVENTION

10 In consideration of the aforementioned problems, the inventors of the present invention conducted extensive studies on whether it is possible to provide a means for predicting whether or not the skin is susceptible to spot formation before a spot is formed in the skin. As a
15 result of conducting a microarray analysis of epidermal RNA from spot sites and non-spot sites in spot model mice in which the skin was irradiated with ultraviolet rays after which irradiation was discontinued so that sunburn-like coloring was discolored followed by waiting briefly
20 before forming pigment spots resembling age spots (M. Naganuma et al., Journal of Dermatological Science 25 (2001) 29-35), the inventors of the present invention found that, in comparison with non-spots sites that were not irradiated with ultraviolet rays and where spot-like
25 pigment spots did not form, expression of the following genes was specifically increased in the epidermis of the spot sites. Thus, it was clearly demonstrated that whether or not skin is susceptible to the formation of spots can be assessed by investigating expression of the
30 following genes in human epidermis.

(1) AK012157 Gene (SEQ. ID NO. 1)

Only the base sequence of this gene is known, and there are no reports describing its function (Meth. Enzymol. 303, 19-44 (1999)). Although a gene having about 80% homology with this gene (S74257: SEQ. ID NO. 3) has been reported to be related to cancer invasion and metastasis in rats (Oncogene, 1994, 9 (12), 3591-3600),

there are no reports on the correlation between this gene and skin pigment. Moreover, although a gene having about 70% homology with mouse gene AK012157 is known to exist in humans (human FLJ21763 gene (SEQ. ID NO. 2)), there 5 are also no reports describing the correlation between this gene and skin pigment.

(2) MCP-2 (Monocyte Chemoattracting Protein 2)

This protein is a type of chemokine (family of small cytokines having molecular weights of about 10,000). 10 Although another member of this family, MCP-1, has been reported to cause non-tumor-forming melanoma cells to form tumors via attraction of monocytes (Journal Immunol. Jun 1; 166(11): 6483-6490), there are no reports on its correlation with skin pigment.

15 (3) Gene Group for which Expression is Known to be Increased by Interferon (hereinafter referred to as "Gene Group 1")

a) Chemokine-Related Genes

20 Mcp9 (small inducible cytokine B subfamily (Cys-X-Cys), member 9) (Arthritis Rheum 2002 Oct; 46(10): 2730-41);

Mcp10 (small inducible cytokine B subfamily (Cys-x-Cys), member 10 (J Immunol. 2002 Apr 1; 168(7): 3195-204);

25 b) Signal Transfer-Related Gene

Isg15 (Interferon-stimulated protein (15 kDa) isg15 (Ubiquitin-like)) (Genes Dev 2003 Feb 15; 17(4): 455-460);

30 Usp18 (ubiquitin specific protease 18) (J. Biol. Chem. 2002 Mar 22; 277(12): 9976-9981);

c) Antivirus-Related Gene

Oas12 (2'-5'-oligoadenylate synthase-like OASL2 (IFN induced)) (J. Interferon Cytokine Res 2002 Sep; 22(9): 981-993);

35 Gbp2 (IFN induced guanylate nucleotide binding protein 2 gbp2 (antivirus)) (J. Interferon Cytokine Res 1998 Nov; 18(11): 977-985);

Gtpi (GTPase; interferon- γ induced GTPase (19440); Ifi47 (interferon gamma inducible protein, 47 kDa (GTP-binding motif) (J. Immunol. 1992 May 15; 148(10): 3275-81);

5 Igtp (GTPase; interferon gamma induced GTPase igtp) (Infect Immun. 2002 Dec; 70(12): 6933-9); Tgtp (GTPase; T-cell specific GTPase (IFN gamma)) (J Leukoc Biol. 1995 Mar; 57(3): 477-83).

An interferon reactive element is present in the 10 promoter region of genes for which expression is increased by interferon, and the expression of these genes is increased as a result of binding thereto STAT-1 (signal transducers and activators of transcription) being activated by phosphorylation (Free Radical Biology 15 & Medicine 2000; 28(9): 1430-1437; Exp Dermatol 1999; 8: 96-108). Thus, the aforementioned Gene Group (1) can be said to be a gene group for which expression is increased in the presence of phosphorylated STAT-1 that has been activated by phosphorylation. As is actually shown in 20 Fig. 6, phosphorylated STAT-1 has also been shown to be expressed at high levels at spot sites.

(4) Other Genes Having Known Functions (hereinafter to be referred to as "Gene Group 2")

25 a) Keratin-Related Gene

Sprr2A (small proline-rich protein 2A) (Mamm. Genome 2003; 14(2): 140-148);

Krt2-6b (keratin complex 2, basic, gene 6a) (Genomics 1998; 53(2): 170-183);

b) Cell Cycle-Related Gene

30 Cdk5rap2 (CKK5 regulatory subunit associated protein 2) (Neuron. 2003 Apr 10; 38(1): 33-46);

Mef2C (myocyte enhancer factor 2C) (Brain Res Mol Brain Res. 2001 Dec 16; 97(1): 70-82);

c) Oxidation-Reduction-Related Gene

35 Gsta4 (glutathione S-transferase, alpha 4) (J. Biol. Chem. 2002 May 17; 227(20): 17892-17900);

d) Bone-Related Gene

Osf2 (osteoblast specific factor (facilin I-like))
(Protein Expr Purif 1995 Jun; 6(39): 305-311);

e) Extracellular matrix (ECM)-Related Gene
Tnc (Tenascin C) (Matrix Biol 2000 Dec; 19(7): 581-
5 596);

f) Insulin-Related Gene
Igfbp6 (Insulin-like growth factor binding protein
6) (Mol. Cell. Endocrinol. 1994; 104(1): 57-66);

g) Cyclosporin-Related Gene
Ppicap (peptidylprolyl isomerase C (cyclophylin C)-
10 associated protein) (Proc Natl Acad Sci USA. 1993 Jul 15;
90(14): 6815-9);
MCP-6 (Mast cell protease 6) (J. Biol. Chem. 1991
Feb 25; 266(6): 3847-3853).

15 (5) Unknown Function Gene Group (hereinafter to be
referred to as "Gene Group 3")
Mm. 74656 Gene (GenBank Acc: Aa519023)
There are no reports describing a correlation with
skin pigment for any of the gene groups of (3). Thus, it
20 is extremely surprising that the expression of these
genes is increased in association with spots.
In a first perspective thereof, the present
invention provides a skin test method for predicting the
formation of spots. This method is characterized in that
25 skin is judged to be susceptible to the formation of
spots in the case expression of MCP2 in epidermis is
increased as compared with normal expression in the
epidermis.
In a preferable aspect thereof, the formation of
30 spots is caused by UVB radiation.
In a more preferable aspect thereof, the increase in
the expression of MCP2 in epidermis is determined by
measuring the amount of MCP2 in the epidermis.
In a more preferable aspect thereof, the measurement
35 is carried out by ELISA or RIA using antibody specific to
MCP2 in the epidermis.
In a more preferable aspect thereof, the increase in

the expression of MCP2 in the epidermis is determined by measuring the amount of mRNA encoding MCP2 extracted from the epidermis.

5 In a more preferable aspect thereof, the measurement of the mRNA is carried out by a polymerase chain reaction method.

10 In a second perspective thereof, the present invention provides a method for screening for a spot formation inhibitory factor and/or spot removal factor. This method is characterized by evaluating a candidate compound for the ability to inhibit the expression and/or activity in the epidermis of MCP2, and select an MCP2 inhibitor having this inhibitory ability as a spot formation inhibitory factor and/or spot removal factor.

15 In a preferable aspect thereof, this method further comprises the application of the MCP2 inhibitor having inhibitory ability to a spot formation model animal, and selecting an inhibitor that has a spot formation inhibitory effect and/or spot removal effect.

20 In a third perspective thereof, the present invention provides another skin test method for predicting the formation of spots. This method judges that skin is susceptible to the formation of spots in the case the expression in the epidermis of a polynucleotide 25 consisting of the base sequence shown in SEQ. ID NO. 2 (human FLJ21763 gene), or a polynucleotide consisting of a sequence of at least 50, preferably at least 100, more preferably at least 200 and particularly preferably at least 400 contiguous nucleotides therein, or the expression in the epidermis of a polynucleotide capable 30 of hybridizing under highly stringent conditions to a polynucleotide consisting of the base sequence shown in SEQ. ID NO. 2 (human FLJ21763 gene), a polynucleotide consisting of the base sequence shown in SEQ. ID NO. 1 (mouse AK012157 gene), a polynucleotide consisting of the base sequence shown in SEQ. ID NO. 3 (rat S74257 gene), or a polynucleotide consisting of at least 50, preferably 35

at least 100, more preferably at least 200, and particularly preferably at least 400 contiguous nucleotides therein, is increased as compared with normal expression in the epidermis. Human FLJ21763 gene and rat 5 S74257 gene are genes that exhibit a high degree of homology with mouse AK012157 gene (having homologies of about 70% and about 80%, respectively). Comparisons among mouse AK012157 gene, human FLJ21763 gene and rat S74257 gene are shown in Figs. 1 and 2. Hybridization 10 can be carried out by a known method or equivalent method thereto such as the method described in J. Sambrook et al., Molecular Cloning 2nd, Cold Spring Harbor Lab. Press, 1989. Highly stringent hybridization conditions refer to conditions in which, for example, sodium 15 concentration is about 10 to 40 mM and preferably about 20 mM, and the temperature is about 50 to 70°C and preferably about 60 to 65°C.

In a preferable aspect, the formation of the spots is caused by UVB radiation.

20 In a more preferable aspect, the increase in the expression of any of the aforementioned polynucleotides in the epidermis is determined by measuring the amount of mRNA complementary to any of the polynucleotides extracted from the epidermis.

25 In a more preferable aspect, the measurement of the mRNA is carried out by the polymerase chain reaction method.

30 In a fourth perspective thereof, the present invention provides another method for screening for a spot formation inhibitory factor and/or spot removal factor. This method evaluates the ability of a candidate consisting to inhibit the expression in the epidermis of a polynucleotide consisting of the base sequence shown in SEQ. ID NO. 2 (human FLJ21763 gene) or the expression of 35 a polynucleotide consisting of a sequence of at least 50, preferably at least 100, more preferably at least 200 and particularly preferably at least 400 contiguous

nucleotides therein, or the expression in the epidermis of a polynucleotide consisting of the base sequence shown in SEQ. ID NO. 2 (human FLJ21763 gene), a polynucleotide consisting of the base sequence shown in SEQ. ID NO. 1 (mouse AK012157 gene), a polynucleotide consisting of the base sequence shown in SEQ. ID NO. 3 (rat S74257 gene), or a polynucleotide capable of hybridizing under highly stringent conditions to a polynucleotide consisting of at least 50, preferably at least 100, more preferably at 10 least 200 and particularly preferably at least 400 contiguous nucleotides therein, and selecting an inhibitor having the inhibitory ability as a spot formation inhibitory factor and/or spot removal factor.

In a more preferable aspect, this method 15 additionally comprises application of the inhibitor having the inhibitory ability to a spot formation model animal to select an inhibitor having a spot formation inhibitory and/or spot removal effect.

In a fifth perspective thereof, the present 20 invention provides another skin test method for predicting the formation of spots. This method judges skin to be susceptible to the formation of spots in the case the expression in the epidermis of a gene for which expression is increased in the presence of phosphorylated 25 STAT-1 (signal transducers and activators of transcription) and interferon increases as compared with normal expression in the epidermis. Here, spots refer to light brown to deep brown flat spots appearing in the skin. Spots mentioned with respect to spot model mice 30 primarily indicate pigment spots resembling age spots.

In a preferable aspect, the gene in the epidermis for which expression is increased in the presence of phosphorylated STAT-1 and interferon is a gene encoding a protein selected from the group consisting of Mcp9, 35 Mcp10, Isg15, Usp18, Oas12, Gbp2, Gtpi, Ifi47, Igtp and Tgtp.

In a sixth perspective thereof, the present

invention provides another skin test method for predicting the formation of spots that judges skin to be susceptible to the formation of spots in the case expression in the epidermis of a gene encoding a protein selected from the group consisting of Sprr2A, Krt2-6b, Cdk5rap2, Mef2C, Gsta4, Osf2, Tnc, Igfbp6 and Ppicap is increased as compared with normal expression in the epidermis.

In a seventh perspective thereof, the present invention provides another skin test method for predicting the formation of spots that judges skin to be susceptible to the formation of spots in the case expression of MCP-6 (mast cell protease 6) is increased as compared with normal expression in the epidermis.

Preferably, the formation of spots is caused by UVB radiation.

In a preferable aspect, the increase in the expression of any of the above genes in the epidermis is determined by measuring the amount of the above proteins in the epidermis.

More preferably, the measurement is carried out by ELISA or RIA using an antibody specific to the protein.

In another preferable aspect, the increase in the expression of any of the above genes in the epidermis is determined by measuring the amount of the mRNA that encodes the above proteins extracted from the epidermis. Preferably, measurement of the mRNA is carried out by the polymerase chain reaction method.

In an eighth perspective thereof, the present invention provides still another method for screening for a spot formation inhibitory factor and/or spot removal factor that evaluates the ability of a candidate compound to inhibit expression of the above genes and/or the activity of proteins which are expression products thereof to select an inhibitor having that inhibitory ability as a spot formation inhibitory factor and/or spot removal factor.

In a preferable aspect, this method comprises application of an inhibitor having the above inhibitory ability to a spot formation model animal to select an inhibitor having a spot formation inhibitory and/or spot removal effect.

In a ninth perspective thereof, the present invention provides still another skin test method for predicting the formation of skin spots that judges skin to be susceptible to the formation of skin spots in the case the expression in the epidermis of a polynucleotide capable of hybridizing under highly stringent conditions to a polynucleotide consisting of the base sequence shown in SEQ. ID NO. 1 (Mm. 74656) is increased as compared with normal expression in the epidermis.

Preferably, the formation of spots is caused by UVB radiation.

In a preferable aspect, the increase in the expression of the above polynucleotide in the epidermis is determined by measuring the amount of mRNA complementary to that polynucleotide extracted from the epidermis.

Preferably, determination of the mRNA is carried out by the polymerase chain reaction method.

In a tenth perspective thereof, the present invention provides still another method for screening for a spot formation inhibitory factor and/or spot removal factor that evaluates the ability of a candidate compound to inhibit the expression in the epidermis of a polynucleotide capable of hybridizing under highly stringent conditions to a polynucleotide consisting of the base sequence shown in SEQ. ID NO. 1 (Mm. 74656), and selects an inhibitor having the inhibitory ability as a spot formation inhibitory factor and/or spot removal factor. Hybridization can be carried out by a known method or equivalent method thereto such as the method described in J. Sambrook et al., Molecular Cloning 2nd, Cold Spring Harbor Lab. Press, 1989. Highly stringent

hybridization conditions refer to conditions in which, for example, sodium concentration is about 10 to 40 mM and preferably about 20 mM, and the temperature is about 50 to 70°C and preferably about 60 to 65°C.

5 According to the present invention, a skin test method can be provided for predicting the formation of spots.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Fig. 1 is a comparison of mouse AK012157 gene, human FLJ21763 gene and rat S74257 gene.

Fig. 2 is a continuation of Fig. 1.

Fig. 3 shows the expression of AK012157 gene and MCP-2 gene in the epidermis and dermis of spot model mice by PCR.

15 Fig. 4 shows the expression of AK012157 gene in the epidermis of spot model mice by *in situ* hybridization.

Fig. 5 shows the expression of MCP-2 in the epidermis of spot model mice by immunohistostaining.

20 Fig. 6 shows differences in the expression of major signal proteins between spot sites and normal sites as determined by Western blotting.

BEST MODE FOR CARRYING OUT THE INVENTION

As has been described above, there have been no reports describing a correlation with skin pigment for mouse AK012157 gene or rat S74257 gene and human FLJ21763 gene, which exhibit high degrees of homology thereto, or the aforementioned Gene Groups (1) to (3). In addition, there are also no reports describing a correlation with skin pigment for mouse or human MCP-2. As a result of 25 conducting microarray analyses on RNA originating in the epidermis of spot sites and normal sites of spot model mice subjected to ultraviolet radiation, the inventors of the present invention found that the expressions of AK012157 gene, MCP-2 gene and genes of the above- 30 mentioned Gene Groups (1) to (3) were specifically increased in the epidermis of spot sites as compared with at non-spot sites. Thus, it was surmised that the 35

formation of spots could be predicted by using these genes as indicators. In particular, increases in the expression of AK012157 gene and MCP-2 gene were observed in the spot model mice not only during UV radiation, but were also observed during the discoloration period when the brown color of the epidermis began to discolor due to UV radiation following completion of UV radiation, and during the pigment spot formation period when spots subsequently began to appear and be formed. Since the expression of these genes in the epidermis of the spot model mice also increases during the discoloration period, if genes homologous with these genes were used as indicators, the future formation of spots could be predicted during the discoloration period before spots have formed.

Spot Model Mice

Spot model mice can be produced as described in the previously listed publication by M. Naganuma et al, op. cit. In short, about 7 week old mice are irradiated with ultraviolet rays about 3 times a week for about 8 weeks at an intensity of about 99 mJ/cm² under an ultraviolet light source (Toshiba FL-SE: UVB). Uniform pigmentation of the skin (browning of the skin) is observed during this UV radiation period. This pigmentation nearly completely disappears about 2 weeks after UV radiation has been discontinued (discoloring period). Subsequently, small, light brown pigment spots having a diameter of about 2mm or less (so-called age spot-like spots) began to appear (pigment spot appearance and formation period).

Skin Test Method for Predicting Spot Formation

The present invention provides a skin test method for predicting the formation of spots in skin, and preferably human skin. This method judges skin to be susceptible to spot formation in the case expression in the epidermis of MCP-2 gene, human FLJ21763 gene, or a polynucleotide capable of hybridizing under highly

stringent conditions to human FLJ21763 gene, mouse AK012157 gene or rat S75257 gene, or a gene selected from the group consisting of Mcp9, Mcp10, Isg15, Usp18, Oas12, Gbp2, Gtpi, Ifi47, Igtp and Tgtp, Sprr2A, Krt2-6b, 5 Cdk5rap2, Mef2C, Gsta4, Osf2, Tnc, Igfbp6 and Ppicap, is increased as compared with normal expression in the epidermis. The evaluation criterion may be, for example, judging skin to be susceptible to spot formation if the expression in the epidermis of MCP-2 gene, human FLJ21763 10 gene, a polynucleotide capable of hybridizing under highly stringent conditions to human FLJ21763 gene, mouse AK012157 gene or rat S74257 gene, or a gene selected from the group consisting of Mcp9, Mcp10, Isg15, Usp18, Oas12, Gbp2, Gtpi, Ifi47, Igtp and Tgtp, Sprr2A, Krt2-6b, 15 Cdk5rap2, Mef2C, Gsta4, Osf2, Tnc, Igfbp6 and Ppicap, is increased by at least 10%, at least 20%, at least 30%, at least 50%, at least 70% or at least 100% in comparison with the expression of those genes in a control epidermis. The skin to be tested may be, for example, 20 skin of the face, neck, limbs or any other portion of the skin that is susceptible to the formation of spots or for which there is concern over the formation of spots. The normal epidermis that is free of spot formation, namely the control epidermis, may be epidermis from the same 25 individual that is, for example, not likely to be exposed to ultraviolet rays or from a site that is relatively resistant to the formation of skin spots such as the abdomen or thigh.

The present invention is also a skin test method for 30 predicting the formation of spots in the skin, and preferably human skin, that judges skin to be susceptible to the formation of spots in the case expression in the epidermis of a polynucleotide capable of hybridizing under highly stringent conditions to MCP-6 or Mm. 74656 35 gene is increased as compared with normal expression in the epidermis. The evaluation criteria may be, for example, judging skin to be susceptible to the formation

of spots if expression in the epidermis of the aforementioned polynucleotide is increased by at least 10%, at least 20%, at least 30%, at least 50%, at least 70% or at least 100% in comparison with expression in a 5 control epidermis.

Hybridization can be carried out by a known method or equivalent method thereto such as the method described in J. Sambrook et al., Molecular Cloning 2nd, Cold Spring Harbor Lab. Press, 1989. Highly stringent hybridization 10 conditions refer to conditions in which, for example, sodium concentration is about 10 to 40 mM and preferably about 20 mM, and the temperature is about 50 to 70°C and preferably about 60 to 65°C.

Increases by the aforementioned genes in the 15 epidermis are determined by, for example, measuring the amount of protein encoded by the genes in the epidermis. For example, an increase in the expression of MCP-2 in the epidermis is determined by measuring the amount of MCP-2 in the epidermis. Preferably, this measurement 20 uses a specific antibody to the aforementioned protein, and can be carried out by various known methods in the industry, such as immunostaining methods using fluorescent substances, pigments or enzymes, Western blotting or immunoassay methods such as ELISA and RIA. 25 In addition, increases in expression can also be determined by extracting RNA from the epidermis and measuring the amount of mRNA that encodes the gene. Extraction of mRNA and measurement of the amount thereof are carried out by known methods in the industry, and for 30 example, quantification of RNA is carried out by the quantitative polymerase chain reaction (PCR) method.

Expression in the epidermis of a polynucleotide capable of hybridizing under highly stringent conditions to the aforementioned genes can be determined by 35 extracting RNA from the epidermis and measuring the amount of mRNA corresponding to the polynucleotide. For example, expression in the epidermis of human FLJ21763

gene or a polynucleotide capable of hybridizing under highly stringent conditions to human FLJ21763 gene, mouse AK012157 gene or rat S74257 gene can be determined by extracting RNA from the epidermis and measuring the 5 amount of mRNA corresponding to the polynucleotide. Extraction of mRNA and measurement of an amount thereof are known in the industry, and for example, quantification of RNA is carried out by a quantitative polymerase chain reaction (PCR) method.

10 As has been previously described, the present invention is based on the finding that, as a result of conducting a microarray analysis of respective epidermal RNA from spot sites and non-spot sites in spot model mice irradiated with ultraviolet rays, the expression of 15 AK012157 gene, MCP-2 gene or a gene of Gene Groups (1) to (3) is specifically increased in the epidermis of spot sites as compared with non-spot sites. Thus, it is surmised that a medicament could be developed that 20 inhibits the formation of spots and/or removes formed spots by using as an indicator inhibition of the expression of the above genes in the epidermis and/or the activity of gene products thereof in the form of the aforementioned proteins, for example, inhibition of the expression of MCP-2 gene in the epidermis and/or the 25 activity of MCP-2, inhibition of the expression of human FLJ21763 gene in the epidermis, or inhibition the expression of a polynucleotide capable of hybridizing under highly stringent conditions to human FLJ21763 gene, mouse AK012157 gene or rat S74257 gene.

30 Thus, the present invention provides a pharmaceutical or skin external composition comprising an inhibitor for inhibiting expression of the aforementioned genes as a spot formation inhibitory factor and/or spot removal factor. The composition as claimed in the 35 present invention is able to prevent the formation or remove spots in the skin.

Examples of inhibitors for inhibiting the activity

of MCP-2 include amino terminal-cleaved MCP-2 having chemokine antagonist activity which lacks the NH₂-terminal amino acid sequence equivalent to amino acid No. 1, Nos. 1 to 2, Nos. 1 to 3, Nos. 1 to 4 or Nos. 1 to 5 of the 5 naturally-occurring MCP-2 described in Japanese Unexamined Patent Publication (Kohyo) No. 2001-518296.

In addition, MCP-2 inhibitors include CCR-1, -3 or -5 receptor antagonists known to be bonded by MCP-2. CCR-3 receptor antagonists are described in, for example, 10 Japanese Unexamined Patent Publication (Kokai) No. H11-14782, Japanese Unexamined Patent Publication (Kohyo) No. 2002-512957, Japanese Unexamined Patent Publication (Kokyo) No. 2002-512960, Japanese Unexamined Patent 15 Publication (Kohyo) No. 2002-530374, Japanese Unexamined Patent Publication (Kohyo) No. 2002-512957 and Japanese Unexamined Patent Publication (Kohyo) No. 2003-510248.

Specific examples of these CCR-3 receptor antagonists include:

20 N-[1-(S)-[4-(3,4-dichlorobenzyl)piperazin-1-ylmethyl]-2-methylpropyl]-4-methylbenzamide dihydrochloride;

N-[1-(S)-[4-(3,4-dichlorobenzyl)piperazin-1-ylmethyl]-2,2-dimethylpropyl]-4-methylbenzamide dihydrochloride;

25 N-[1-(S)-[4-(3,4-dichlorobenzyl)piperazin-1-ylmethyl]-2-methylpropyl]-4-methylbenzamide dihydrochloride;

N-[1-(R)-[4-(3,4-dichlorobenzyl)piperazin-1-ylmethyl]-2-methylpropyl]-4-(2-aminoethyl)benzamide 30 dihydrochloride;

N-[1-(R)-[4-(3,4-dichlorobenzyl)piperazin-1-ylmethyl]-2-methylpropyl]-5-methylthiophene-2-carboxamide hydrochloride;

35 1-[1-(R)-[4-(3,4-dichlorobenzyl)piperazin-1-ylmethyl]-2-methylpropyl]-3-(3-methoxyphenyl) urea;

1-[1-(R)-[4-(3,4-dichlorobenzyl)piperazin-1-ylmethyl]-2-methylpropyl]-3-(3-methoxyphenyl) urea;

(S)-ethyl-2-(4-methylbenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(5-dimethylaminonaphthalene-1-sulfonylamino)-3-(4-hydroxyphenyl)propionate;
5 (S)-ethyl-2-(naphthalene-2-sulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(thiophene-2-sulfonylamino)-3-(4-hydroxyphenyl)propionate;
10 (S)-ethyl-2-(quinoline-8-sulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(2,4,6-trimethylbenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
15 (S)-ethyl-2-(4-bromobenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(4-chlorobenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
20 (S)-ethyl-2-(4-methoxybenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-methanesulfonylamino)-3-(4-hydroxyphenyl) propionate;
25 (S)-ethyl-2-[2-(E)-styrylsulfonylamino]-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(3-trifluoromethylbenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
30 (S)-ethyl-2-(2,5-dichlorothiophene-3-sulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(2-bromobenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
35 (S)-ethyl-2-[5-(2-pyridyl)thiophene-2-sulfonylamino]-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(1,3-dimethyl-5-chloro-2-pyrazoline-4-sulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(4-biphenylsulfonylamino)-3-(4-hydroxyphenyl)propionate;
40 (S)-ethyl-2-(2-nitro-4-methoxybenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(2,5-dichlorobenzenesulfonylamino)-3-[4-

(2,5-dichlorobenzenesulfonyloxy)phenyl]propionate;
(S)-ethyl-2-(2,4-difluorobenzenesulfonylamino)-3-[4-(2,4-difluorobenzenesulfonyloxyphenyl)propionate;
(S)-ethyl-2-(5-dimethylaminonaphthalene-1-sulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(thiophene-2-sulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(2,4,6-trimethylbenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(4-bromobenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(4-chlorobenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(4-methoxybenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-[2-(E)-styrylsulfonylamino]-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(3-trifluoromethylbenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(2,5-dichlorothiophene-3-sulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(2-bromobenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-[5-(2-pyridyl)thiophene-2-sulfonylamino]-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(2-nitro-4-methoxybenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(2,5-dichlorobenzenesulfonylamino)-3-[4-(2,5-dichlorobenzenesulfonyloxy)phenyl]propionate;
(S)-ethyl-2-(2,5-dichlorothiophene-3-sulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(2-bromobenzenesulfonylamino)-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-(2,5-dichlorobenzenesulfonylamino)-3-[4-(2,5-dichlorobenzenesulfonyloxy)phenyl]propionate;
(S)-ethyl-2-(1-naphthoylamino)-3-(4-nitrophenyl)propionate;

(S)-isopropyl-2-(1-naphthoylamino)-3-(4-nitrophenyl) propionate;

(S)-methyl-2-(1-naphthoylamino)-3-(4-nitrophenyl) propionate;

5 (S)-benzyl-2-(1-naphthoylamino)-3-(4-nitrophenyl) propionate;

(S)-ethyl-2-(1-naphthoylamino)-3-(4-chlorophenyl) propionate;

10 (S)-ethyl-2-benzoylamino-3-(4-hydroxyphenyl) propionate;

(S,S)-ethyl-2-(2-benzylxycarbonylamino-3-phenylpropionylamino)-3-(4-hydroxyphenyl)propionate;

(S,S)-ethyl-2-(N-acetylpyrrolidine-2-benzoylamino)-3-(4-hydroxyphenyl)propionate;

15 (S)-ethyl-2-cyclohexanylamino-3-(4-hydroxyphenyl) propionate;

(S)-ethyl-2-(3,3-diphenylpropionylamino)-3-(4-hydroxyphenyl)propionate;

20 (S)-ethyl-2-(3-phenylpropionylamino)-3-(4-hydroxyphenyl)propionate;

(S)-ethyl-2-[2-(2-naphthyl)acetylamino]-3-(4-hydroxyphenyl)propionate;

(S)-ethyl-2-(4-phenylbutyrylamino)-3-(4-hydroxyphenyl)propionate;

25 (S)-ethyl-2-pentanylamino-3-(4-hydroxyphenyl) propionate;

(S)-ethyl-2-pentanylamino-3-(4-hydroxyphenyl) propionate;

(S)-ethyl-2-(4-benzoylbenzoylamino)-3-(4-hydroxyphenyl)propionate;

30 (S)-ethyl-2-(2-furanyl)amino-3-(4-hydroxyphenyl)propionate;

(S)-ethyl-2-(1-naphthoylamino)-3-(4-hydroxyphenyl) propionate;

35 (S)-ethyl-2-(5-hydroxyindonyl)amino-3-(4-hydroxyphenyl)propionate;

(S)-ethyl-2-piperonylamino-3-(4-hydroxyphenyl)

propionate;
 (S)-ethyl-2-picolinylamino-3-(4-hydroxyphenyl)
propionate;
 (S)-ethyl-2-(3-nitro-4-chlorobenzoylamino)-3-(4-
5 hydroxyphenyl)propionate;
 (S)-ethyl-2-(3-hydroxy-4-nitrobenzoylamino)-3-(4-
hydroxyphenyl)propionate;
 (S)-ethyl-2-(8-quinolinylamino)-3-(4-hydroxyphenyl)
propionate;
10 (S)-ethyl-2-benzoylamino-3-phenylpropionate;
 (S)-methyl-2-benzoylamino-3-(4-hydroxyphenyl)
propionate;
 (S)-benzyl-2-benzoylamino-3-(4-hydroxyphenyl)
propionate;
15 (S)-ethyl-2-benzoylamino-3-(4-methoxyphenyl)
propionate;
 (S)-ethyl-2-tert-butyloxycarbonylamino-3-(4-
nitrophenyl)propionate;
 (S)-ethyl-2-tert-butyloxycarbonylamino-3-(4-
20 aminophenyl)propionate;
 (S)-ethyl-2-benzoylamino-3-(3,5-diido-4-
hydroxyphenyl)propionate;
 (S)-ethyl-2-carboxybenzoylamino-3-(4-hydroxyphenyl)
propionate;
25 (S)-ethyl-2-benzoylamino-3-(1-naphthyl)propionate;
 (±)-ethyl-2-benzoylamino-3-[3-(benzoyloxy)phenyl]
propionate;
 (±)-ethyl-2-benzoylamino-3-(3-
hydroxyphenyl)propionate;
30 (R,S)-ethyl-2-benzoylamino-3-(2-hydroxyphenyl)
propionate;
 (S)-ethyl-2-benzoylamino-3-(4-
aminophenyl)propionate;
 (S)-ethyl-2-benzoylamino-3-(4-
35 nitrophenyl)propionate;
 (S)-ethyl-2-(2-phenylacetylamino)-3-(4-
hydroxyphenyl) propionate;

(S)-ethyl-2-benzoylamino-3-(3-indoyl)propionate;
(±)-ethyl-2-(benzoylamino)-2-phenylacetate;
(±)-ethyl-2-(benzoylamino)-4-phenylbutyrate;
(S)-ethyl-2-(1-naphthoylamino)-3-(4-hydroxyphenyl)
5 propionate;
(S)-ethyl-2-benzoylamino-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-cyclohexanylamino-3-(4-hydroxyphenyl)propionate;
10 (S)-ethyl-2-(1-naphthoylamino)-3-(4-hydroxyphenyl)propionate;
(S)-benzyl-2-benzoylamino-3-(4-hydroxyphenyl)propionate;
(S)-ethyl-2-benzoylamino-3-(4-methoxyphenyl)propionate;
15 (S)-ethyl-2-benzoylamino-3-(1-naphthyl)propionate;
(±)-ethyl-2-benzoylamino-3-[3-(benzoyloxy)phenyl]propionate;
20 (±)-ethyl-2-benzoylamino-3-(3-hydroxyphenyl)propionate;
(R,S)-ethyl-2-benzoylamino-3-(2-hydroxyphenyl)propionate;
(S)-ethyl-2-benzoylamino-3-(4-nitrophenyl)propionate;
25 (±)-ethyl-2-(benzoylamino)-2-phenylacetate;
(±)-ethyl-2-(benzoylamino)-4-phenylacetate;
(S)-ethyl-2-(1-naphthoylamino)-3-(4-nitrophenyl)propionate;
30 (S)-ethyl-2-(1-naphthoylamino)-3-(4-hydroxyphenyl)propionate; and,
(S)-ethyl-2-benzoylamino-3-(4-nitrophenyl)propionate.

35 Examples of CCR-1 receptor antagonists include the compounds described in International Publication WO 97/24325; WO 98/38167 by Pfizer, Inc., WO 97/44329 by

Teijin Co., Ltd.; WO 98/04554 by Banyu Pharmaceutical Co., Ltd.; WO 98/27815, WO 98/25604, WO 98/25605, WO 98/25617 and WO 98/31364 by Merck & Co., Inc.; WO 98/02151 and WO 99/37617 by LeukoSite, Inc.; WO 99/37651 and WO 99/37619 by LeukoSite, Inc.; US Provisional Patent Application No. 60/021,716 (filed on July 12, 1996); US Patent Application No. 09/146,827 and 09/148,236 (filed on September 4, 1998); Hesselgesser et al., *J. Biol. Chem.* 273(25): 15687-15692 (1998); and Howard et al., *J. Medicinal Chem.* 41(13): 2184-2193 (1998).

In addition, an example of a CCR-5 receptor antagonist is described in Japanese Unexamined Patent Publication (Kohyo) No. 2002-543186.

A pharmaceutical or skin external composition of the present invention is applied in the form of, for example, an aqueous solution, oily liquid, other type of solution, milky liquid, cream, gel, suspension, microcapsules, powder, granules, capsules or solid preparation. After preparing in these forms using conventionally known methods, they can be applied, attached, sprayed, injection, consumed or inserted into the body in the form of a lotion, milky lotion, cream, ointment, salve, poultice, aerosol, water-oil two-layer system, water-oil-powder three-layer system, injection, oral preparation (e.g., tablets, powders, granules, pills, syrup, lozenges) or suppositories. The aforementioned inhibitor can be contained in this composition at, for example, 0.001 mM to 1 M, preferably 0.01 to 100 mM and more preferably 0.1 to 10 mM, based on the total amount of the composition with any particular limitations as a spot formation inhibitory factor and/or spot removal factor.

Among these drug forms, lotions, milky lotions, creams, ointments, salves, poultices, aerosols and other skin external preparations are suitable for the object of the present invention. Furthermore, the skin external preparations listed here include prescription pharmaceuticals, over-the-counter pharmaceuticals (such

as ointments) and cosmetics (such as facial washes, milky liquids, creams, gels, essences (beauty washes), facial packs, facial masks and other basic cosmetics, foundations, lipstick and other makeup cosmetics, as well 5 as oral cavity cosmetics, fragrant cosmetics, hair cosmetics and body cosmetics). A pharmaceutical or skin external preparation of the present invention is particularly suitably applied as a spot preventive cosmetic.

10 Conventionally known vehicles and fragrances as well as oils, surfactants, antiseptics, metal ion chelating agents, water-soluble polymers, thickeners, pigments and other powdered components, ultraviolet protectors, moisturizers, antioxidants, pH regulators, cleansers, 15 desiccants or emulsifiers and so forth are suitably incorporated in a pharmaceutical or skin external preparation of the present invention corresponding to the desired drug form. Moreover, other pharmacologically active components can be incorporated into a 20 pharmaceutical or skin external preparation of the present invention within a range that does not impair the expected effects as a result of incorporation.

Spot Formation Inhibitory Factor and/or Spot Removal Factor Screening Method

25 The present invention additionally provides a method for screening for a spot formation inhibitory factor and/or spot removal factor. This method is characterized by evaluating a candidate compound for the ability to inhibit the expression and/or activity in the epidermis 30 of the aforementioned genes such as MCP2, or the ability to inhibit the expression of a polynucleotide capable of hybridizing under highly stringent conditions to human FLJ21763 gene, mouse AK012157 gene or rat S74257 gene, and select an inhibitor having this inhibitory ability as 35 a spot formation inhibitory factor and/or spot removal factor.

In a preferable aspect thereof, the aforementioned

screening method comprises applying an inhibitor having the inhibitory ability to a spot model animal, and selecting an inhibitor having a spot formation inhibitory effect and/or spot removal effect.

5 A process for confirming the spot formation inhibitory effect and/or spot removal effect of the inhibitor can be carried out by using a model animal such as a spot model animal. Examples of animals that can be used as models other than mice include rats, rabbits and
10 various other animals. In a preferable aspect thereof, a solution such as an aqueous solution of the inhibitor is prepared, and then repeatedly applied to the skin of the spot model animal followed by evaluation of spot formation to judge the presence or absence of the above
15 effects.

The following provides a more detailed explanation of the present invention using a specific example. Furthermore, the present invention is not limited thereto.

20 Production of Spot Model Mice

Spot model mice were produced as described in M. Naganuma et al, op cit. In short, 7 week old mice were irradiated with ultraviolet rays 3 times a week for 8 weeks (UV radiation period) at an intensity of 99 mJ/cm² 25 under an ultraviolet light source (Toshiba FL-SE: UVB). The animals underwent a discoloring period from age 15 to 23 weeks (until about 8 weeks following completion of UV radiation period), a pigment spot appearance period from age 23 to 35 weeks (about 18 to 30 weeks after completion 30 of UV radiation period), and a pigment spot formation period from age 35 to 52 weeks (about 30 to 47 weeks after completion of UV radiation period).

Sampling of RNA from Skin

35 The entire layer of skin was sampled from the back of each mouse followed by removal of the fatty layer and separation of the epidermis by heating. RNA was extracted from the epidermis using ISOGEN (Nippon Gene,

manufacturer's recommended protocol) and purified using RNeasy (Qiagen). The dermis was cut into 1 centimeter squares, frozen in liquid nitrogen and crushed followed by extraction of RNA using ISOGEN (Nippon Gene, 5 manufacturer's recommended protocol) and purification using RNeasy (Qiagen). The resulting RNA was phoresed in TBE agarose gel and stained with SYBR Green (Molecular Probes) to confirm quality and the degree of purification.

10 Samples during the UV radiation period were collected from about 10 week old mice, and controls for these samples were collected from about 14 week old mice. Samples during the discoloring period were collected from about 20 week old mice, and controls for these samples 15 were collected from about 20 week old mice. Samples during the pigment spot formation period were collected from about 42 week old mice, and controls for these samples were collected from about 40 week old mice.

Reaction of Microarray Samples

20 Gene chips manufactured by Affymetrix Japan were used for microarray analyses.

Affymetrix Sample Preparation:

The reaction was carried out according to the Affymetrix recommended protocol. 10 μ g of RNA were 25 reacted with 100 pmol of oligo-dT primer (24 mer) (Sigma) for 10 minutes at 70°C. Subsequently, 4 μ l of 5x first strand reaction buffer, 2 μ l of 0.1 M DTT, 1 μ l of 10 mM dNTP and 2 μ l of Superscript II (all Invitrogen products) were added and reacted for 1 hour at 42°C. 91 μ l of 30 RNase-free water, 30 μ l of 5x second strand reaction buffer, 3 μ l of 10 mM dNTP mix, 1 μ l of 10 U/ μ l E. coli DNA ligase, 14 μ l of 10 U/ μ l E. coli DNA polymerase and 1 μ l of 2 U/ μ l RNaseH (all Invitrogen products) were then 35 added and reacted for 2 hours at 16°C. Moreover, 2 μ l of T4 DNA polymerase (10 U/ μ l) (Invitrogen) were added and

after reacting for 5 minutes at 16°C, 10 µl of 0.5 M EDTA were added to stop the reaction. Cleanup was carried out using Phase Lock Gels (Qiagen) and eluted into 12 µl of RNase-free water. Biotin-added cRNA was produced by an 5 in vitro transcription reaction from the resulting cDNA (Enzo, Farmingdale), and then purified using RNeasy (Qiagen). After fragmenting the cRNA for 35 minutes at 94°C in a fragmentation buffer, the resulting fragmented cRNA was used for gene chip hybridization. After washing 10 the chip, the data was read using a scanner manufactured by Affymetrix.

Analysis Results

As a result of comprehensively analyzing about 9,000 types of genes, the expression of AK12157 gene, MCP-2 15 gene and Gene Groups (1) to (3) were found to be remarkably increased in epidermis of the spot model mice as compared with the epidermis of control mice. Particularly interestingly, increased expression of AK012157 gene and MCP-2 gene was observed not only during 20 the UV radiation period, but was also continuously observed during the discoloration period, when the brown color of the epidermis began to discolor due to the UV radiation following completion of UV radiation, and during the subsequent pigment spot formation period when 25 spots began to appear and be formed. Since the expression of these genes was also increased during the discoloration period prior to the formation of spots in the epidermis of the model mice, the use of these genes as indicators would make it possible to predict the future formation of spots even during the discoloration 30 period prior to spot formation. Those results are shown in Tables 1 and 2 below.

Table 1 Fluctuation Rates of Each Gene Based on Control

	UV radiation period	Discoloration period	Pigment spot formation period
MCP-2	4.7	13.8	3.2
AK012157	5.3	5.5	6.4

(Expression ratio based on a value of 1 for a normal site or non-UV irradiated site. Average values for n = 2 for the UV radiation and discoloration periods, and n = 3 for the pigment spot formation period.)

Table 2

Gene Name	Spot sites/non-spot sites (n=4)
Mcp9	3.9
Mcp10	3.6
Isg15	4.1
Usp18	2.2
Oas12	4.1
Gbp2	3.3
Gtpi	3.2
Ifi47	2.8
Igtp	2.7
Tgtp	2.6
Sprr2A	23.3
Krt2-6b	3.4
Cdk5rap2	12.4
Mef2C	4.8
Gsta4	3.0
Osf2	2.9
Tnc	2.9
Igfbp6	2.9
Ppicap	2.6
Mcp-6	1.8
Mm. 74656	2.9

10

RT-PCR

1 μ g each of the RNA collected from epidermis (epidermis and dermis of a spot site and non-spot site) was reacted for 10 minutes at 70°C with 100 pmol of oligo-dT primer (24 mer) (Sigma) (total volume: 20 μ l).

15

Subsequently, 4 μ l of 5x first strand reaction buffer, 2

5 μ l of 0.1 M DTT, 4 μ l of 2.5 mM dNTP mix, and 1 μ l of Superscript II (all Invitrogen products) were added and reacted for 1 hour at 42°C. Finally, an elongation reaction was carried out for 10 minutes at 70°C to prepare cDNA templates. 10 5 μ l of rTaq 10x buffer, 3 μ l of 25 mM MgCl₂, 5 μ l of 2.0 mM dNTP mix, 0.5 μ l of rTaq (all Toyobo), 33.5 μ l of ddH₂O, 1 μ l of each cDNA template, and 1 μ l of each sense and antisense primer (refer to the sequences shown below) at 20 mM each were all added (total volume: 50 μ l) followed by carrying out the PCR reaction (30 cycles consisting of 94°C for 2 minutes (94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute) and finally 72°C for 10 minutes).

15 MCP-2 Primer Sequences:

Sense TTCTTTGCCTGCTGCTCATA (SEQ ID NO. 4)
Antisense GACAAGGATGAGAAAACACG (SEQ. ID NO. 5)

AK012157 Primer Sequences:

Sense ACTCCGGCTCCTTCACTATG (SEQ. ID NO. 6)
Antisense CTTTGGAAATGAGGACTTGGA (SEQ. ID NO. 7)

20 Finally, the PCR products were electrophoresed with agarose gel containing 1.5% ethidium bromide to obtain a band of about 300 bp. This result is shown in Fig. 3. As is clear from Fig. 3, AK012157 gene and MCP-2 gene were significantly expressed at a spot site of the epidermis, but were observed to be hardly expressed at 25 all at a non-spot site of the epidermis. In addition, there were no differences in expression for either gene in the dermis.

30 In Situ Hybridization (ISH)

After fixing mouse epidermal tissue with 10% neutral formalin and embedding in paraffin, tissue section slides were produced according to ordinary methods. The slides were then subjected to an in situ hybridization reaction using the HX System Automated Slide Processor 35 manufactured by Ventana Japan (Protocol: Japan Open Blue

8.0). The tissue sections were subjected to pretreatment consisting of blocking and protease treatment, followed by hybridizing for 6 hours at 68°C with 500 ng of a Dig-labeled riboprobe produced from the AK012157 sequence using T7 polymerase. The hybridization product was then washed, and after reacting with Anti-Dig-alkaline phosphatase and washing, the product was stained using NBT/BCIP substrate followed by sealing and observing with a light microscope.

Those results are shown in Fig. 4. As is clear from Fig. 4, AK012157 gene was only expressed significantly in a spot site of the epidermis, and was observed to be hardly expressed at all in a non-spot site of the epidermis.

15 Immunohistochemical Staining (IHC)

After fixing mouse epidermal tissue with neutral formalin and embedding in paraffin, tissue sections were produced in accordance with ordinary methods. After then treating for 15 minutes with 1% H₂O₂, the sections were blocked for 30 minutes and reacted overnight with 1/50-diluted anti-mouse MCP2 antibody (R&D Systems). This was detected by tyramide sensitization (TSA System, Perkin-Elmer). After washing, the sections were reacted with secondary antibody with HRP and then washed, followed by reacting with FITC-labeled tyramide, washing, embedding and observed with a fluorescent microscope.

Those results are shown in Fig. 5. As is clear from Fig. 5, MCP-2 gene was observed to be significantly expressed at a spot site in the epidermis, and was observed to hardly be expressed at all at a non-spot site in the epidermis.

Comparison of Expression of Major Signal Transfer Protein Groups

When epidermal proteins were detected at spot sites and normal sites in spot model mice, and the expressed amounts of the major signal protein groups shown in Fig. 6 were investigated by Western blotting, phosphorylated

STAT1 exhibited a particularly large difference in expression between spot sites and normal sites. This result coincide with the finding that an interferon-induced gene group (Gene Group (1)) demonstrates increased expression at spot sites.

5

INDUSTRIAL APPLICABILITY

The present invention is able to provide a skin test method for predicting the formation of spots in the skin.